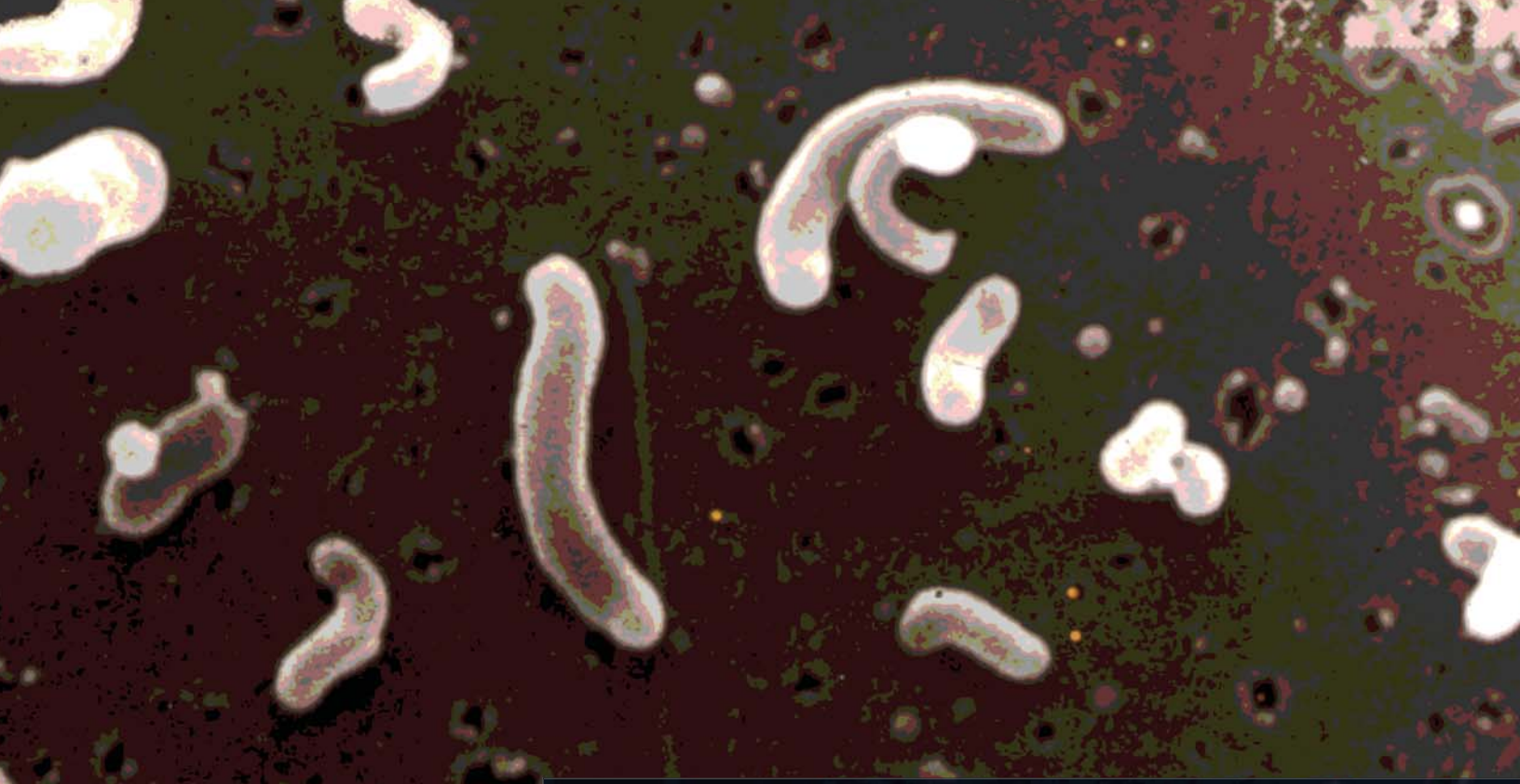
So Many Microbes, So Little Time, and So Little Money

Needs and Challenges in Developing a Global Network of Biological Resource Centers

BY D. JAY GRIMES



When microbiologists first applied the tools of their trade to the oceans of the world, they quickly learned that water samples contained variable numbers of bacteria, depending on sample location and the method of measurement. Estuarine water samples were routinely found to contain greater numbers of culturable bacteria than coastal ocean water samples, which, in turn, contained greater numbers than samples from the abyssal ocean. In general, microscopic counts always exceeded culture medium counts, for any given sample, and activity measurements often suggested a greater bacterial presence than indicated by the culture counts. Claude ZoBell was one of the first marine microbiologists to make such observations (ZoBell and Upham, 1944), and over the years many other investigators repeated and expanded these findings. Typical were the observations of Grimes et al., (1984) who reported that acridine orange direct counts (AODCs) of bacteria in seawater samples usually exceeded plate counts on heterotrophic plating media by four to six orders of magnitude, and they discussed reasons for the discrepancy. Data analysis presented by Meyer-Reil (1978) also supported these observations, further indicating the common failure of cultural-count data and direct-count data to exhibit any correlation. Illustrative of this discrepancy is the data presented in Figure 1.

Over the years, many explanations were given for these bacterial concentration discrepancies, and they can be summarized in two ways: (1) the medium or conditions (e.g., temperature, pressure, salinity, pH, Eh) being used for cultivation could not support the growth of all physiological types of bacteria present in the sample; and (2) although still intact, many cells observed in the sample were moribund or dead and therefore incapable of further growth. Both of these hypotheses are partially correct, but for reasons not fully appreciated when they were first proposed.

Selection of growth medium and conditions is very important. There is no single medium that will support the growth of all heterotrophic bacteria, let alone autotrophs—be they phototrophic or lithotrophic. Even with a suite of media and conditions, many organisms will go uncounted. During the past two decades, it has become clear that viable bacteria that cannot be cultured on media normally supportive of their growth also account for some fraction of the total count that will not grow under laboratory conditions; these bacteria are thought to be dormant and have been called somnicells (Roszak and Colwell, 1987). Both somnicells and organisms not yet cultured (i.e., not yet discovered) fail to grow on plating media, and therefore contribute to the discrepancies.

Another reason given for discrepancies between total and plate counts (intact moribund or dead cells) was long thought to be the principal explanation. While some cells in any given system are presumably moribund or dead, this factor can no longer be supported as the principal reason. For example, Tabor and Neihof (1984) used a variety of direct methods (synthetically active bacteria, INT [2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride] reduction, and microautoradiographic determination of uptake-active organisms) to demonstrate that 25 to over 85 percent of cells in Chesapeake Bay water samples (as determined by AODCs) were indeed viable. Clearly, viable but nonculturable bacteria—both dormant and yet to be cultured—can now be considered responsible for the major portion of the total count-plate count discrepancies in most systems.

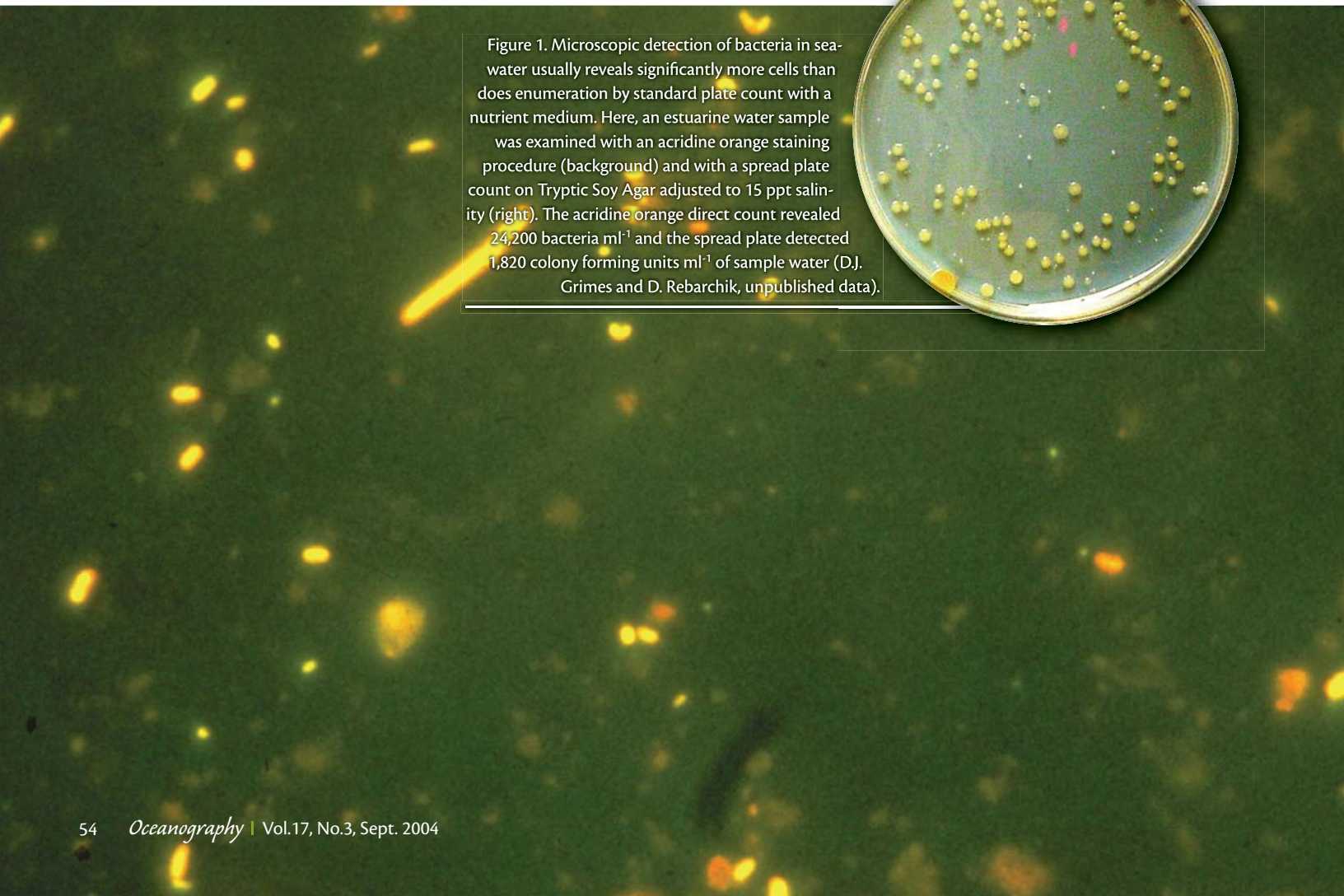


Figure 1. Microscopic detection of bacteria in seawater usually reveals significantly more cells than does enumeration by standard plate count with a nutrient medium. Here, an estuarine water sample was examined with an acridine orange staining procedure (background) and with a spread plate count on Tryptic Soy Agar adjusted to 15 ppt salinity (right). The acridine orange direct count revealed 24,200 bacteria ml^{-1} and the spread plate detected 1,820 colony forming units ml^{-1} of sample water (D.J. Grimes and D. Rebarchik, unpublished data).

UNDERSTANDING AND CAPTURING MICROBIAL DIVERSITY

Recent evidence suggests there are many "yet to be cultured" microorganisms from all three domains—*Bacteria*, *Archaea*, and *Eukarya*—present in the biosphere. Their discovery is awaiting developments in microbial culture technology so that they too can be isolated and grown in sufficient quantity to be described. Bull et al. (1992) estimated the total number of bacterial species on Earth to be 40,000, and Tiedje (1994) pointed to different lines of evidence that suggest between 300,000 and 1 million species of bacteria inhabit the soil. More recently, Curtis et al. (2002) estimated that the sea contains 2 million different bacteria and that a ton of soil contains 4 million. *Bergey's Manual of Systematic Bacteriology* (4 volumes containing 2,784 pages) presently lists 3,100 species of bacteria (Holt, 1984). The 2nd edition of *Bergey's* is now in preparation (only the first of 5 volumes is in print [Garrity et al., 2001]) and it is estimated that, given an annual increase of over 200 new prokaryotic species, all 5 volumes will contain over 7,100 species (G.M. Garrity, personnel communication, 2004). GenBank now lists approximately 30,000 species, based on 16S rRNA sequences, of which most have not been cultured. If one accepts the Curtis et al. (2002) hypothesis, *Bergey's Manual*, in contemporary format (circa 775 species per volume, excluding genetic databases), could ultimately reach 2,500 volumes in length! Clearly, new technologies are facilitating new discovery of microbes at unprecedented rates. The National Science Foundation, in recognition of this vast discovery opportunity, has now held four competitions for "microbial observatories." The purpose of this program is to discover novel microorganisms and microbial consortia, communities, activities, and other novel properties, and to study their roles in diverse environments (http://www.nsf.gov/pubs/2002/nsf02118/nsf02118.htm).

The oceans of the world account for much of microbial diversity and contain countless nano- and picoplankton, the majority of which are yet to be cultured (Schmidt et al., 1991). For example, the cold water *Archaea*, first detected in seawater samples collected at depths ranging from 100 to 500 meters (DeLong 1992; Fuhrman et al., 1992), comprise a substantial percentage of the total microbial biomass present in oceans throughout the world (DeLong et al., 1994). Recently, DeLong and Pace (2001) estimated that *Archaea* represent 20 percent or more of all microbial cells in the oceans. These *Archaea* have not been isolated in pure culture, nor have they been grown and phenotypically described. They have been detected only by PCR amplification, characterization, and classification of their ribosomal RNA. In 1996, Woese notes that approximately 300 archaeal species had been described in the literature; he believes that this number will expand significantly (C.R. Woese, personal communication, 1996). Boone observed that methanogen discovery alone has accounted for 5 to 10 new species per year for the last 10 years (D.R. Boone, personal communication, 1996). It is doubtful that all of these yet to be cultured *Archaea* and *Bacteria* are dormant in their respective habitats; some must be active and involved in biogeochemical cycling and possibly disease processes in ways that remain unexplored. This hypothesis is supported by extensive circumstantial evidence, for example, data provided by vital staining techniques such as the AODC, direct viable counting (Kogure et al., 1979), and INT staining (Baker and Mills, 1982). More recently, flow cytometry has become a useful tool to identify and sort active cells—both culturable and yet to be cultured (Rinaldo et al., 2002). Based on their presumed relative abundance, and on community structure shifts in response to environmental disturbance (White, 1993), yet to be cultured *Archaea* and *Bacteria* no doubt transform vast quantities of geochemicals in the biosphere.

Recently, the extent of this situation has been made even more obvious with the emergence of major taxa of *Bacteria* for which there are few (or even no!) cultured members (Hugenholtz et al., 1998; Gordon and Giovannoni, 1996). Major lineages such as the *Verrucomicrobia*, *Acidobacterium*, SAR11, SAR83, SAR86 and others are now known to occur in environments around the world (Figure 2), yet they have almost no connection with a physiological database; accordingly, virtually no information is available as to their role in biosphere. Distribution of these yet to be cultured bacteria is truly ubiquitous; it includes novel forms such as the SAR324 cluster found in the lower surface layers of both the Atlantic and Pacific Oceans (Wright et al., 1997) and the SAR11 clade which dominates surface bacterioplankton communities in the world's oceans (Morris et al., 2002). Clearly, the molecular data indicate large numbers of unusual and undiscovered bacteria exist in the oceans—perhaps three orders of magnitude beyond known species. Equally clear is our present inability to predict function from genomic information. And adding to

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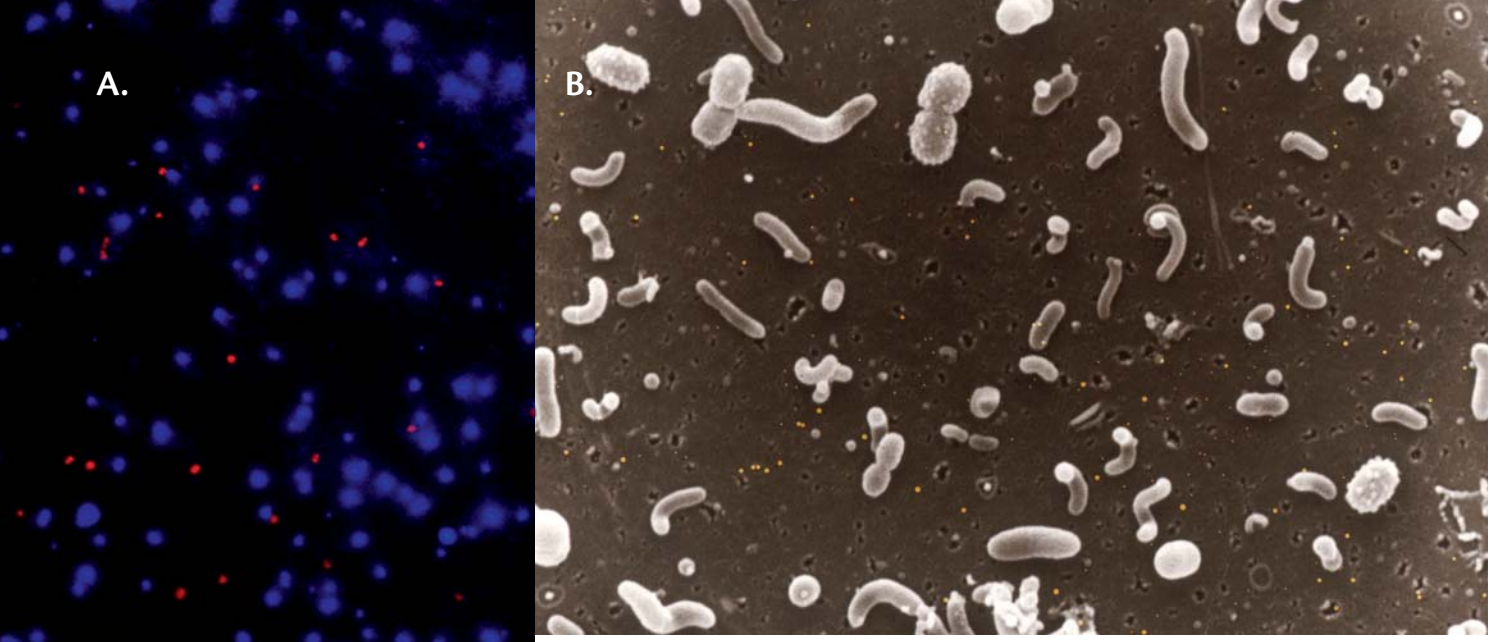

Figure 2. The oceans of the world abound with microorganisms, most of which have yet to be discovered. In any given photomicrograph or electron micrograph of seawater, the viewer can be assured of seeing unknown microbes. In plate (A) a photomicrograph of bacteria in seawater is visualized by means of epifluorescence microscopy; yet-to-be cultured SAR86 cells glow red from a specific rRNA probe (copyright Marion LeClerc, MBRI, 2000). In plate (B), scanning electron microscopy reveals many unusual shapes of oceanic bacteria. Photos courtesy of Ed DeLong, Monterey Bay Aquarium Research Institute. http://www.mbari.org/news/news_releases/2000/sep15_delong_fig.html

this dilemma is the recent, astounding genomic sequencing effort by Craig Venter's group that resulted in the discovery of 1,800 new bacterial species from the Sargasso Sea (Venter et al., 2004). We know there are many "bugs" out there, but we do not know what they are doing, nor to we have an easy or economic way to isolate, grow, describe, and maintain them. Indeed, it was only through laborious and time-consuming methods that a member of the SAR11 clade was recently cultured (Rappe et al., 2002) and named *Pelagibacter ubique*. Nevertheless, now is the time to begin counting, characterizing, classifying, and collecting new bacteria, culturable or not; the scientific community needs a census of all marine life and it needs ready access to all representative species in order to further basic understanding. In turn, such a census and understanding will allow the industrial community to discover and market new applications.

The German Collection of Microorganisms and Cell Cultures, the DSMZ, estimates that it costs them $2,500-3,000 (USD) to add a bacterial culture to its collection. Given that an estimated 1 to 2 million bacteria remain to be isolated from nature, their acquisition and incorporation into the DSMZ would cost at least $2.5 to 6 billion. The American Type Culture Collection (ATCC) estimates that it costs $5,000-10,000 (depending on the type and quality of the material—tissue cultures, microorganisms, databases, etc.) to add new items to its collection, when the costs of quality control, validation, long-term preservation and global distribution are taken into consideration. Accessioning all of the undiscovered bacteria into the ATCC could cost as much as $20 billion! Based on these numbers, which in general do not account for complimentary genomic and proteomic database development, the conclusion is obvious. No single collection or even country in the world can easily afford to isolate, characterize and accession into a culture collection all of the extant bacterial species. New public-private partnerships are needed to more equitably and efficiently distribute this expanding need and responsibility.

Because scientists will continue to isolate new microbes, describe their place in nature, document their attributes, and make them available to all users—science, health, agribusiness, biotechnology, and industrial—a new paradigm for safekeeping and distribution is needed. One solution to this huge burden and responsibility is to share the costs of procurement, maintenance and distribution across several countries. Recently, the Organisation for Economic Co-operation and Development (OECD), the Paris based body comprised of the world's leading country economies, concluded a study of Biological Resource Centers (BRCs) and their role in the advance-

ment of life sciences and biotechnology. The OECD study report (OECD, 2001) called for the establishment of a global network of BRCs, through which biological materials and related information would flow.

WHAT ARE BRCs?

As defined by the OECD (OECD, 2001):

Biological resource centers are an essential part of the infrastructure underpinning life sciences and biotechnology. They consist of service providers and repositories of the living cells, genomes of organism, and information relating to heredity and the functions of biological systems. BRCs contain collections of culturable organisms (e.g., micro-organisms, plant, animal and human cells), replicable parts of these (e.g., genomes, plasmids, viruses, cDNAs), viable but not yet culturable organisms, cells and tissues, as well as databases containing molecular, physiological and structural information relevant to these collections and related bioinformatics.

This BRC concept goes far beyond that of a traditional bacterial culture collection; BRCs will become the future, definitive source of all authenticated biological material and information.

WHY ARE BRCs ESSENTIAL?

Living organisms—cells, genes, gene products—are the essential raw materials of biological investigations and are crucial for the advancement of biotechnology, health sciences, and research and development in the life sciences. Without biological resources and the related information or databases that describe them, life scientists cannot pursue their research, companies focused on biotechnology cannot develop and market products, and health researchers cannot elucidate and cure diseases of humans, animals, and plants. On the other hand, when biological resources are made available through reputable and continuously available sources, all of humankind benefits. Given that enormous sums are invested in extracting organisms and their genes from nature and elucidating the genetic and functional molecular elements of those living resources, it is essential that biological resources not only be preserved but also be available for use. And simply having the genome sequenced is not a replacement for having the actual organism in culture; the genome is a "parts list" and the biology and ecology of the microbe is considerably more complicated and less understood. By making biological materials and information of guaranteed identity, quality, and security available to all users, BRCs will serve an essential infrastructural function for scientific investigation and research and development.

WHY ESTABLISH A GLOBAL BRC NETWORK?

The establishment of a global BRC network would provide the framework within which coordination, harmonization and quality assurance could be provided for the preservation and elucidation of biodiversity, elucidation of relationships between organisms, and international exchange of biological resources. This would enhance the services provided to the global community by BRCs beyond what existing international frameworks could achieve. Specifically, a global BRC network would add value by achieving: (1) two-way communication and linkages between scientific needs and government policies (the main reason that the OECD initiated this effort); (2) an international framework for regulatory initiatives, either directly or through the appropriate national and international authorities; (3) international cooperation to help prevent inappropriate use of biological resources, for example, terrorism; (4) a mechanism by which countries that cannot reach the standards required of BRCs can build capacity and in the meantime become part of a global system; and (5) enhance efficiency, as a global BRC network would reduce redundancies and improve transparency and thus, over time, help participants to harness resources for meaningful research and economic development.

WHAT ARE THE CHALLENGES?

The development, expansion, and survival of individual BRCs and the establishment of a global BRC network face many challenges (Table 1). These include those posed by the molecular revolution (genomics and proteomics), accelerating efforts to conserve biodiversity, funding uncertainties that threaten stability of current culture collections, the need

TABLE 1. The Challenges and Opportunities Facing Creation of a Global BRC.

The molecular revolution data overload
Accelerating efforts to conserve biodiversity
Funding uncertainties that threaten stability
Collections at risk
Need for adequate Quality Assurance/ Quality Control (QA/QC)
Constraints on access within countries and across international borders
Cooperation with customs
Private industry's need to protect investments
Import and export regulations
Intellectual property rights
Personnel safety issues—biosafety
Misuse—security issues
Seamless inquiries and data transfer

for universally accepted quality assurance, constraints on access to biological resources within countries and across international borders, private industry's need to protect investments, import and export regulations, intellectual property rights, biosafety issues that range from laboratory accidents to intentional misuse of select agents, and ethical concerns about the uses of genes and other biological resources. These challenges are far too large for any single nation to solve in an increasingly globalized world, and a shared international approach is needed. Only very few, large national centers are currently able to perform a comprehensive role and to provide access to diverse organisms and databases.

WHAT ACTION IS NEEDED?

The OECD report (OECD, 2001) recommended the establishment of an international system for accreditation that would ensure the quality and stability of a system of national BRCs linked together as a global network. According to the report, a global network would foster international cooperation and economic development, while meeting a worldwide need for access to quality biological resources. In line with OECD tradition, the work on BRCs was guided by individual "lead nations" which also provided material support. The development of the BRC report (OECD, 2001) and the formulation of an international consensus were led by Japan, with strong support from others, particularly the United States. In follow up to the report (OECD, 2001), the implementation phase is being guided by France (Schwartzenberg, 2001), again with strong support from other countries (Table 2). It is hoped that OECD member country ministers (both participants and nonparticipants, see Table 2) will agree to the framework for a global system of accreditation for BRCs and give their backing to completing the establishment of the international system.

Although a global BRC presents a major challenge for international cooperation, such a network would greatly improve the conditions under which biological materials are preserved and exchanged. How this challenge is met will affect the life sciences and biotechnology for many years to come. It is a challenge that calls for the full support of governments, the scientific community, and the collective international private sector. There are many microbes, and other biological resources, which await discovery and description, and these resources need a safe and secure home once they are discovered. Resources already discovered and described also need to be safe and secure for future understanding and application. As Dixon (1996) correctly notes in his commentary written eight years ago, the vast number of microbes that remain to be discovered holds great promise and excitement for "those who are starting now." Since 1996, the new revelations from genomics have only added emphasis to Dixon's statement. Clearly, there is little time to waste before the nations of the world unite to achieve the economy of scale that a global BRC network would provide.

TABLE 2. Countries Participating in the Task Force on Biological Resource Centers for the OECD Working Party on Biotechnology.

OECD Members[1]	Non-OECD Members
Austria	Brazil
Belgium	Burkina Faso
Canada	Colombia
Czech Republic	Laos
France	Malaysia
Germany	Philippines
Hungary	Senegal
Italy	Thailand
Japan	**Observers**
Korea	China[2]
Mexico	
Netherlands	
Poland	
Portugal	
Spain	
Switzerland	
United Kingdom	
United States	

[1]Member countries not participating in the Task Force on BRCs: Australia, Denmark, Finland, Greece, Iceland, Ireland, Luxembourg, New Zealand, Norway, Slovak Republic, Sweden, and Turkey.
[2]Currently an official observer to the OECD Committee for Scientific and Technological Policy (CSTP).

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Table 3. Tasks Under Investigation and Development by Focus Groups of the Task Force on Biological Resources Centers.

Focus Group 1

Accreditation system and quality criteria

1. Framework for accreditation will be developed by the Task Force
2. BRC accreditation will be the responsibility of each participating national government
3. Accreditation will focus on quality
4. Two levels of accreditation – one at the international level and one at the national level based on domains or biological material (e.g., plants, microbes, animals, humans, and databases)
5. Accreditation will be subject to regular review

Focus Group 2

International linkages

1. Design of a general framework for information retrieval and exchange
2. Need to link to other initiatives

Focus Group 3

Biosecurity, biosafety, funding, and harmonization of legal issues

1. Monitor select agents
2. Develop funding plans
3. Monitor ethics, Intellectual Property Rights (IPR), and laws

Center for Research Resources, the National Science Foundation, and the U.S. Department of Energy's Office of Biological and Environmental Research. Dawn Rebarchik provided the materials for Figure 1. Salomon Wald provided the initial opportunity for OECD participation.

REFERENCES

Baker, K.H., and A.L. Mills. 1982. Determination of the number of respiring *Thiobacillus ferrooxidans* cells in water samples by using combined fluorescent antibody - 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride staining. *Appl. Environ. Microbiol*. 43:338-344.

Bull, A.T., M. Goodfellow, and J.H. Slater. 1992. Biodiversity as a source of innovation in biotechnology. *Ann. Rev. Microbiol.* 46:219-252.

Curtis, T.P., W.T. Sloan, and J.W. Scannell. 2002. Estimating prokaryotic diversity and its limits, *Proc. Natl. Acad. Sci.* (USA) 99:10494-10499.

DeLong, E.F. 1992. *Archaea* in coastal marine environments. *Proc. Natl. Acad. Sci.* (USA) 89:5685-5689.

DeLong, E.F., and N.R. Pace. 2001. Environmental diversity of bacteria and *Archaea*. *Syst. Biol.* 50: 470-478.

DeLong, E.F., K.Y. Wu, B.B. Prézelin, and R.V.M. Jovine. 1994. High abundance of *Archaea* in Antarctic marine picoplankton. *Nature* (Lond.) 371: 695-697.

Dixon, B.. 1996. So many microbes, so little time. *Bio/Technology.* 14:122.

Fuhrman, J.A., K. McCallum, and A.A. Davis. 1992. Novel major archaebacterial group from marine plankton. *Nature* (Lond.) 356:148-149.

Garrity, G.M., D.R. Boone, and R.W. Castenholz, eds. 2001. *Bergey's Manual of Systematic Bacteriology,* 2nd ed., v. 1. Springer-Verlag, New York, 721 pp.

Gordon, D.A., and S.J. Giovannoni. 1996. Detection of stratified microbial populations related to *Chlorobium* and *Fibrobacter* species in the Atlantic and Pacific Oceans. *Appl. Environ. Microbiol.* 62:1171-1177.

Grimes, D.J., F.L. Singleton, and R.R. Colwell. 1984. Allogenic succession of marine bacterial communities in response to pharmaceutical waste. *J. Appl. Bacteriol.* 57:247-261.

Holt, J.G., ed. 1984. *Bergey's Manual of Systematic Bacteriology*, v. 1-4. Williams & Wilkins, Baltimore, 2,784 pp.

Hugenholtz, P., B.M. Goebel, and N.R. Pace. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* 180:4,765-4,774.

Kogure, K., U. Simidu, and N. Taga. 1979. A tentative direct microscopic method for counting living marine bacteria. *Can. J. Microbiol.* 25:415-420.

Meyer-Reil, L.A.. 1978. Autoradiography and epifluorescent microscopy combined for the determination of number and spectrum of actively metabolizing bacteria in natural waters. *Appl. Environ. Microbiol.* 36:506-512.

Morris, R.M., M.S. Rappe, S.A. Connon, K.L. Vergin, W.A. Siebold, C.A. Carlson, and S.J. Giovannoni. 2002. SAR11 clade dominates ocean surface bacterioplankton communities. *Nature.* 420:806-810.

OECD (Organisation for Economic Co-operation and Development). 2001. *Biological Resource Centres: Underpinning the Future of Life Sciences and Biotechnology.* OECD Publications, Paris, France, 66 pp.

Rappe, M.S., S.A. Connon, K.L. Vergin, and S.J. Giovannoni. 2002. Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature.* 418:630-633.

Rinaldo, A., A. Martin, K.Cavender-Bares, and S.W. Chisholm. 2002. Cross-scale ecological dynamics and microbial size spectra in marine ecosystems. *Proc. Roy. Soc. Lond.* B 269:2051-2059.

Roszak, D.B., and R.R. Colwell. 1987. Survival strategies of bacteria in the natural environment. *Microbiol. Rev.* 51:356-379.

Schmidt, T.M., E.F. DeLong, and N.R. Pace. 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. *J. Bacteriol.* 173: 4,371-4,378.

Schwartzenberg, R.G. 2001. Installation du comite consultative des resources biologiques. Discours de M. Roger-Gerard Schwartzenberg, minister de la Recherche, Paris, France. [Online] Available at: http://www.recherche.gouv.fr/discours/2001/ccrbiod.htm [accessed July 20, 2004].

Tabor, P.S., and R.A. Neihof. 1984. Direct determination of activities for microorganisms of Chesapeake Bay populations. *Appl. Environ. Microbiol.* 48:1,012-1,019.

Tiedje, J.M. 1994. Microbial diversity: of value to whom? *ASM News.* 60:524-525.

Venter, J.C., K. Remington, J.F. Heidelberg, A.L. Halpern, D. Rusch, J.A. Eisen, D. Wu, I. Paulsen, K.E. Nelson, W. Nelson, D.E. Fouts, S. Levy, A.H. Knap, M.W. Lomas, K. Nealson, O. White, J. Peterson, J. Hoffman, R. Parsons, H. Baden-Tillson, C. Pfannkoch, Y-H. Rogers, and H.O. Smith. 2004. Environmental genome shotgun sequencing of the Sargasso Sea. *Sciencexpress* (March 4): Publ. Online.

White, D.C. 1993. *In situ* measurement of microbial biomass, community structure and nutritional status. *Phil. Trans. R. Soc.* (Lond) A 344:59-67.

Wright, T.D., K.L. Vergin, P.W. Boyd, and S.J. Giovannoni. 1997. A novel -subdivision proteobacterial lineage from the lower ocean surface layer. *Appl. Environ. Microbiol.* 63:1,441-1,448.

ZoBell, C.F., and H.C. Upham. 1944. *A List of Marine Bacteria Including Descriptions of Sixty New Species*. University of California Press, Berkeley, 292 pp.